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Tryptophane Metabolism, Cancer of the Urinary Bladder and Smoking Habits[1])

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Dedicated to Prof. Dr. Dr. E. Werle on the occasion of his 70th birthday

In order to obtain information on a possible causative connection between the excretion of certain tryptophane metabolites and the etiology of cancer of the urinary bladder and to evaluate the influence of smoking on the etiology of this malignant disease, the following groups were formed:
I. Healthy persons, smokers, II. healthy persons, non-smokers, III. patients (no bladder cancer), smokers, IV. patients (no bladder cancer) non-smokers, V. patients with bladder cancer, smokers. The following tryptophane metabolites were estimated: Kynurenine, 3-hydroxy-kynurenine, 3-hydroxy-anthranilic acid, N'-methyl-nicotinamide.
No significant differences between the study groups could be found by the analysis of variance. However, there was some indication for a larger variation in the group of cancer patients.

Um die Möglichkeit des kausativen Zusammenhanges zwischen der Ausscheidung von bestimmten Tryptophanmetaboliten und der Ätiologie des Harnblasencarcinoms und den Einfluß des Rauchens auf die Entstehung dieser malignen Tumoren zu prüfen, wurden die folgenden Versuchsgruppen gebildet:
I. Gesunde Probanden, Raucher, II. gesunde Probanden, Nichtraucher, III. Patienten (kein Blasen-Carcinom), Raucher, IV. Patienten (kein Blasen-Carcinom), Nichtraucher, V. Patienten mit Blasen-Carcinom, Raucher. Die Konzentrationen der folgenden Metabolite im 24-h-Harn wurden bestimmt: Kynurenin, 3-Hydroxykynurenin, 3-Hydroxyanthranilsäure, N'-Methylnicotinamid.
Es konnte mit der Varianzanalyse keine signifikanten Unterschiede zwischen den Gruppen nachgewiesen werden; jedoch deuten weitere statistische Analysen an, daß die Gruppe der Krebspatienten durch eine größere Variabilität gekennzeichnet ist.

An impressive example for environmental carcinogenesis, which has been known for a long time, is cancer of the urinary bladder. Certain carcinogenic aromatic amines have proved to be the cause of industrial bladder cancer. In the course of investigations which were based on theoretical conceptions endogenous compounds have also been found to be able to produce bladder cancer in mice after local application (Boyland (1)). Furthermore Boyland et al. found, that patients with urinary bladder cancer excrete more carcinogenic endogenous substances than normal persons. These facts represented a coincidence of experimental and clinical findings in cancer research. For the first time, the enhanced production of an endogenous compound had been recognized as a carcinogenic agent. The compounds which proved to be carcinogenic, are 3-hydroxy-kynurenine, 3-hydroxy-anthranilic acid, and 2-amino-3-hydroxy-acetophenone.
The finding of Boyland et al. (2, 3) that the activity of esterases, which split the conjugated forms of the above mentioned compounds is elevated in the urine of patients with bladder cancer, provided additional evidence for the concept of impaired tryptophane metabolism as a cause of urinary bladder cancer in man. For the first time, an hypothesis about the endogenous mechanism of a cancer etiology was proved experimentally. It is thus astonishing, that only relatively few investigations exist on this subject (4). We think that the lack of relevant investigations is due to analytical difficulties.
In retrospective and prospective studies, an epidemiological and statistical connection between the occurrence of bladder cancer and smoking of cigarettes (review see by (5)) has been found. In 1965 Kerr et al. (6) reported on the impairment of tryptophane metabolism by smoking. They measured the excretion of tryptophane metabolites in six subjects before and after smoking and found that after tryptophane loading, the test persons excreted more 3-hydroxy-kynurenine and more 3-hydroxy-anthranilic acid during a smoking period than during abstinence from smoking.
This was in fact a remarkable finding, which proved for the first time a metabolic block in a natural degradation mechanism caused by tobacco smoke constituents.

[1]) Extracts of this paper were read during the conference of the "Deutsche Gesellschaft für Klinische Chemie" and "Deutsche Gesellschaft für Hämatologie", 27th and 28th September 1972 in Höhenried.

These findings are of great importance from the biochemical as well as from the etiological point of view. A confirmation of these findings can give additional evidence for the etiological significance of endogenous compounds in urinary bladder cancer. In earlier investigations we found that cigarette smoke is able to influence tryptophane metabolism (7, 8). We could show that cigarette smoke inhibits kynureninase in vitro, but activates this enzyme in the liver of hamsters exposed to cigarette smoke. The activity of tryptophane pyrrolase is inhibited in vitro and in vivo by application of cigarette smoke.

In light of these preliminary considerations and experimental findings, we decided to reinvestigate with improved methods the excretion of carcinogenic tryptophane metabolites in normal persons and in patients with urinary bladder cancer. We were especially interested in any correlations with smoking habits.

We have already mentioned the suspicion that the lack of reliable clinical-chemical methods for the measurement of the carcinogenic compounds in question has been the cause for the lack of confirming or non-confirming studies on this subject. During preliminary studies on suitable chemical methods we found that several methods which are described in the literature, and which were used earlier by other investigators, did not fullfill the requirements of modern analytical methods with respect to precision and specificity. We directed our attention to the estimation of the carcinogenic compounds 3-hydroxy-kynurenine and 3-hydroxy-anthranilic acid. After having found that none of the methods described in the literature were sufficient for our investigations, we developed a combined enzymatic method for the estimation of 3-hydroxy-kynurenine and 3-hydroxy-anthranilic acid in urine which is extremely specific (9).

The findings which we will present in this paper are very different from the above mentioned findings of other authors. Therefore, we will give a detailed description of materials and methods used.

Materials and Methods

The tryptophane metabolism is dependent on the diet, and as we were looking for possible differences with regard to smoking habits, the need arose to form the following groups:

1. Normal persons, smokers
2. Normal persons, non-smokers
3. Patients without bladder cancer, smokers
4. Patients without bladder cancer, non-smokers
5. Patients with bladder cancer, smokers.

For the purpose of this investigation, smokers were defined as smokers if they smoked regularly or did so during a time period in their life regardless of their present smoking habits. Non-smokers had never smoked regularly in their lives.

Normal persons were volunteers from the hospital staff or persons known to the hospital staff. Patients with bladder cancer were inmates of the urological clinic of the University of Munich, and patients without bladder cancer were inmates of the same hospital. Most of them suffered from bone fractures but they were on the same diet as the bladder cancer patients. Table 1 summarizes sex and age distribution and smoking habits for the 5 study groups.

The original number of persons in groups 3 to 5 was considerably greater, but for reasons which will be mentioned later, a substantial number of the urine specimens could not be analysed because of the lack of stability of 3-hydroxy-anthranilic acid (for details see "chemical methods"). Group 3 and 4 consisted mostly of patients with bone fractures. They were inmates in the same University hospital as the patients of group 5 and received the same diet. Group 5 consisted only of patients with bladder cancer which had been histologically verified in the excised tumor. Histological examinations were performed in the Institute of Pathology, University of Munich.

During the time of this study, we had only two patients with bladder cancer who were non-smokers. The values found in these patients are not included in this study because such a small number would not give reliable results.

Urine was collected during 24 h before surgical treatment. To the first urine portion, 5 ml concentrated HCl (D = 1.19) was added. All urine samples which were more alkaline than pH 6 were discarded for reasons which will be mentioned later. Immediately after collection, each urine sample was portioned into separate containers for the different chemical estimations and kept frozen until used.

Chemical methods

The following tryptophane metabolites were measured: kynurenine, 3-hydroxy-anthranilic acid, 3-hydroxy-kynurenine, and N'-methyl-nicotinamide. 3-Hydroxy-kynurenine and 3-hydroxy-

Tab. 1
Age and sex distribution and smoking of the study groups

Study group nr.		Sex	n	Age (range)	Cigarettes consumed* Daily (mean)	Years
Normal persons	1 smokers	♂	9	28—50	10	8
		♀	13	26—49		
	2 non-smokers	♂	7	26—34	—	—
		♀	6	23—59		
Patients without bladder cancer	3 smokers	♂	18	28—72	23	20
		♀	5	26—54		
	4 non-smokers	♂	3	45—69	—	—
		♀	19	50—71		
Patients with bladder cancer	5 smokers	♂	14	40—71	11	18
		♀	4	45—70		

* In group 1 one person smoked cigars and four smoked pipes in addition.
In group 3 two patients smoked cigars and pipes in addition to cigarettes.
In group 5 one patient smoked pipe, two patients smoked cigars and one patient smoked pipes and cigars.

anthranilic acid were estimated in the free and in the conjugated form.

Kynurenine

This metabolite was evaluated by the method of TOMPSETT (10). The method is based upon alkaline distillation of the urine and the consequent forming of *o*-amino acetophenone. This compound is measured in the distillate by a diazo reaction. In the original method, the distillate is collected in three test tubes. Normally, the third tube shows only traces of colored material after the reaction. In several urine samples, we found an extremely high content of diazo positive material which was still present in the fifth or sixth test tube. Thin-layer chromatography and other methods showed that this material was not all kynurenine. Consequently, the values obtained from these urines which had been collected from persons of all groups, were not included into the interpretation of this study.

3-Hydroxy-kynurenine and 3-Hydroxy-anthranilic acid

At first we tried to estimate both of these substances by column and thin layer chromatography. A satisfactory separation between kynurenine and 3-hydroxy-kynurenine could not be obtained by these methods. Therefore, we developed an enzymatic method, which is based upon the conversion of 3-hydroxy-kynurenine and 3-hydroxy-anthranilic acid by kynureninase (L-kynureninehydrolase, EC 3.7.1.3) and 3-hydroxy-anthranilic acid oxidase (3-hydroxy-anthranilate: O_2 oxidoreductase, EC 1.13.1.6), respectively into a degradation product which can be measured at 340 nm. The amount of the product is equivalent to the amount of 3-hydroxy-kynurenine and 3-hydroxy-anthranilic acid present in the urine (9). This method is absolutely specific for the two mentioned compounds. 3-Hydroxy-anthranilic acid is a very labile compound and can decompose easily in solutions with a pH higher than 6 (11), so no urine samples which were more alkaline than 6 were used for the estimation of 3-hydroxy-anthranilic acid. For hydrolysis of the conjugated compounds, a mixture of β-glucuronidase and arylsulfatase (Boehringer Mannheim) was used.

N'-methylnicotinamide was estimated by the method described by CARPENTER and KODICEK (12).

Biostatistics

As the data contain some gross outliers, the study groups will be characterized by the median instead of the more usual arithmetic mean.

The testing for group differences presents some difficulties with regard to the multitude of hypotheses to be tested: as is well-known an increased number of tests will lead to an increased number of wrong statements. For this reason it was decided to analyse the data by means of an analysis of variance (ANOVA). This helps to keep the overall error for the comparison of the five study groups within certain prespecified bounds, for which we choose the conventional 5%-level. As we were very concerned about not overlooking a real difference (β-error) we decided to admit an $\alpha = 0.05$ for each of the seven variables.

The analyses of variance were performed with the help of a linear model whose fit could be tested by inspection of the so called residual plots.

Some of the distributions were analysed graphically by plotting them into probability paper. The handling of small samples by this technique has been described in detail by L. G. JOHNSON (13).

Results

The median values[2]) for the different study groups concerning the tryptophane metabolites are presented in Table 2 and 3. Group comparisons for each variable with respect to the mean were performed by the overall F-test of an analysis of variance (one way analysis). Inspection of the residuals in normal probability paper revealed that the data situation could be improved by a logarithmic transformation of all data ($X = \log_{10}(X + 1)$). After transformation the residuals showed a sufficiently normal distribution although in some cases the extreme ends left something to be desired.

[2]) The median is an alternative measure for the central tendency of a distribution. It is defined as that point on the scale of observations on each side of which are equal areas under the histogram (the 50th percentile). Thus the median is the middle observation if there is an odd number of cases.

Tab. 2

Excretion of 3-hydroxy-kynurenine and 3-hydroxy-anthranilic acid in free and conjugated form in the urine. Median values, mg/24 h
n = number of persons in the group, n+ = number of assays

Group	n	3-hydroxy-kynurenine				3-hydroxy-anthranilic acid			
		free	n+	total	n+	free	n+	total	n+
1	22	0.41	21	0.52	21	0.32	22	0.56	22
2	13	0.44	13	0.50	13	0.23	13	0.39	13
3	23	0.79	20	1.29	20	0.33	20	0.51	20
4	22	0.33	20	0.48	20	0.22	21	0.33	21
5	18	0.76	14	1.14	13	0.36	16	0.56	14

Tab. 3

Excretion of N'-methyl-nicotinamide, kynurenine and creatinine. Median values
n = number of persons in the group, n+ = number of assays

Group	n	N'-methyl-nicotinamide mg/24 h	n+	Kynurenine mg/24 h	n+	Creatinine g/24 h	n+
1	22	3.26	22	1.32	21	1.50	22
2	13	3.38	13	1.23	13	1.43	13
3	23	1.79	22	1.81	22	1.76	23
4	22	2.78	22	2.42	16	1.39	22
5	18	1.99	18	8.86	13	0.99	18

In none of the five analyses was there a significant F-value, although some single t-test comparisons reach the level of significance.

The compounds 3-hydroxy-kynurenine and 3-hydroxy-anthranilic acid in the free form were analysed in more detail. The groups of cancer patients and smokers from hospital are contrasted in probability paper with logarithmic abscissa (Fig. 1 and 2) as well as the two healthy groups of smokers and non-smokers (Fig. 3 and 4).

It may be derived from Figure 1 that there is some indication for a different distribution of the measurements for the two groups: there is more variation in the group of cancer patients. The difference of the two distributions with respect to their variability, however, could not be shown to be statistically significant (SIEGEL and TUKEY test; $p > 0.10$). The graphical representation of the cases suggest that the different study groups in themselves are very inhomogeneous.

As for normal persons, smokers and non smokers do not differ substantially (Fig. 3 and 4). There is practically no difference with respect to 3-hydroxy-anthranilic acid in patients (Fig. 2). The representation of normal patients suggests that there might be a difference between smokers and non-smokers with respect to the central tendency of the distribution (Fig. 3). The difference, however, might still be explained by chance alone (WILCOXON rang sum test).

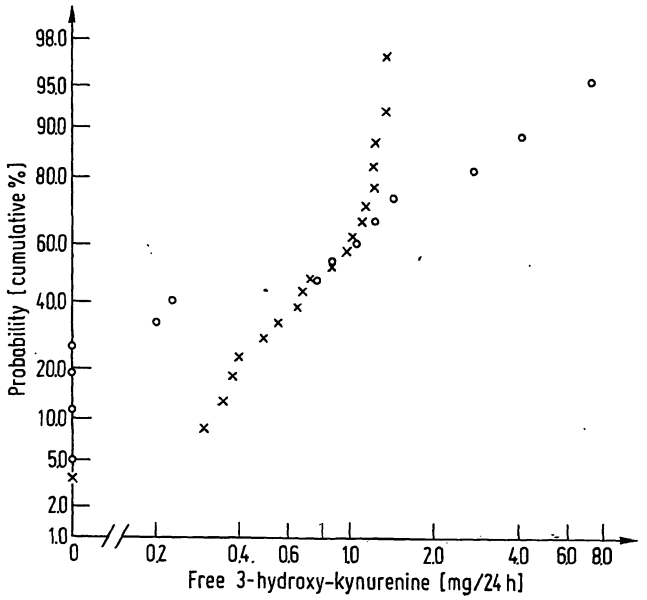

Fig. 1
Excretion of free 3-hydroxy-kynurenine in study groups 3 and 5 (× = patients without bladder cancer, smokers, o = cancer patients, smokers) on probability paper

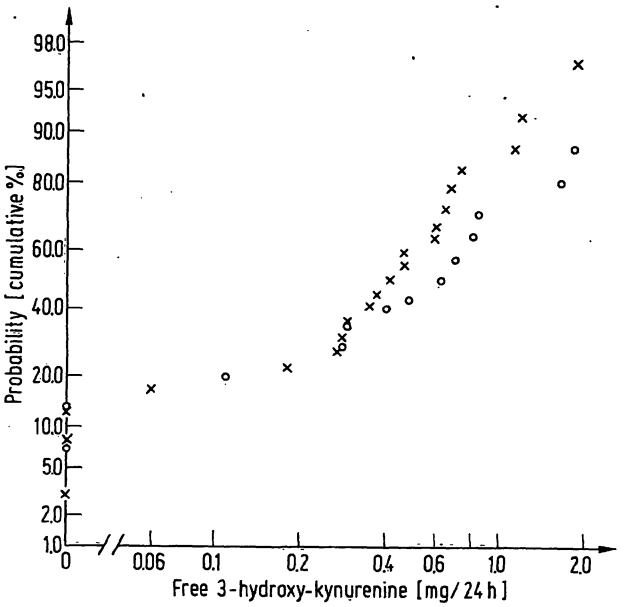

Fig. 3
Excretion of free 3-hydroxy-kynurenine in study groups 1 and 2 (× = normal persons, smokers, o = normal persons, non-smokers) on probability paper

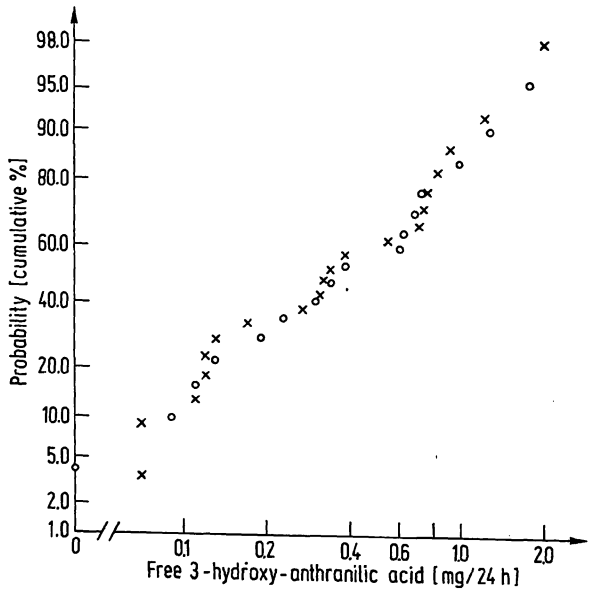

Fig. 2
Excretion of free 3-hydroxy-anthranilic acid in study groups 3 and 5 (× = patients without bladder cancer, smokers, o = patients with bladder cancer, smokers) on probability paper

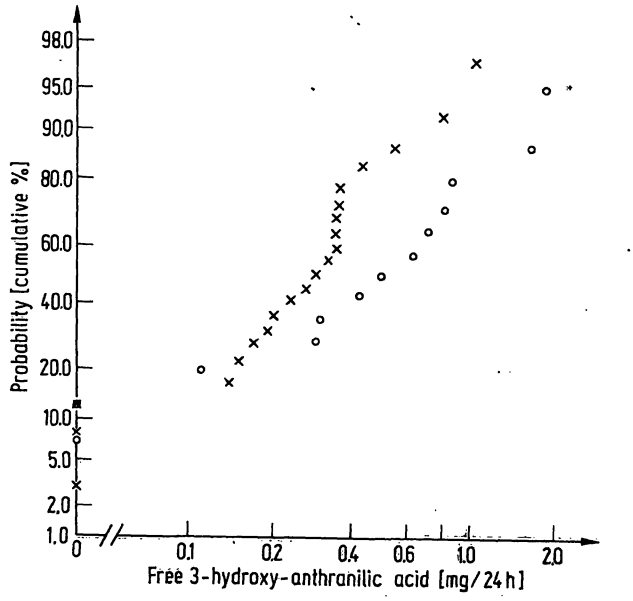

Fig. 4
Excretion of free 3-hydroxy-anthranilic acid in study groups 1 and 2 (× = normal persons, smokers, o = normal persons, non-smokers) on probability paper

Diskussion

The analyses of variance resulted in the verdict of "no significant differences" which — as one should bear in mind — does not imply that there is none. There is much variation in the data which may camouflage real differences. And of course the F-test is less sensitive if the number of groups increases. We adhered to the F-test, however, because we prefer a more conservative decision concerning the null-hypothesis of no group difference.

It may be noted that there exists a high degree of non-homogeneity in most distributions as was displayed by the graphic representation of the data. For the 3-hydroxy-kynurenine variable there is some indication that the group of cancer patients shows a greater variability than the comparable study group of smoking patients.

The findings of BOYLAND et al. (1, 2, 3) and of several other investigators show that patients with bladder cancer excrete more carcinogenic tryptophane metabolites than do normal persons. Our results do not confirm these earlier results. The difference between our findings and those of the above mentioned authors may be based upon the different methods used for the estimation of the tryptophane metabolites. TOMPSETT (10) already observed that the urine of patients with malignant disease of the bladder sometimes contains non specific diazotable volatile amines in appreciable amounts. This confirms our findings with regard to the results of kynurenine estimations mentioned earlier.

The methods used by us for the estimation of 3-hydroxy-kynurenine and 3-hydroxy-anthranilic acid are absolutely specific. They can be controlled by running a control sample of the respective urine with standard substance. In this way an inhibitor or other disturbing substances present in the urine, which may interfer with the assay can be detected.

Our investigations did not produce any indication that a correlation exists between the excretion of certain tryptophane metabolites and the etiology of bladder cancer. In earlier investigations we could show that cigarette smoke may interfere with tryptophane metabolism *in vivo* and *in vitro* (7, 8), but the present findings do not show any significant correlation between cigarette smoking and bladder cancer. Our findings are contrary the the results of KERR et al. (6). This group of authors found a positive correlation between cigarette smoking and excretion of certain tryptophane metabolites. They used the methods criticized above, and moreover, they had only six persons in their study and measured the metabolites after tryptophane loading.

Our results are in accordance with results of BROWN et al. (14) and of ALPEROVITCH (15), who also could not confirm the results of KERR et al. (6). Moreover, we think that measurement of tryptophane metabolism after tryptophane loading may not reflect the true conditions. But in the past, this has been necessary as no methods existed which were sensitive enough to measure the normal excretion.

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